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INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
James	Russell	Vancouver, CANADA

Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Thrombomodulin (THBD) haplotypes predict outcome of patients

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 17 ☐ CD(s), Number

☑ Drawing(s) *Number of Sheets* 13 ☐ Other (specify)

☐ Application Date Sheet. See 37 CFR 1.76

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Respectfully submitted,

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Date March 3/04

TYPED or PRINTED NAME Elaine Weiss

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1. Provisional application for patent cover sheet
2. Credit card payment for $80.00 filing fee
3. Specifications, 17 pages
4. Drawings, 13 pages

THE UNIVERSITY OF BRITISH COLUMBIA

March 4, 2004

Hon. Commissioner of Patents and Trademarks,
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Dear Sir:

Re: Provisional Application for "Thrombomodulin (THBD) haplotypes predict outcome of patients."

UBC file no: 04-098

Enclosed please find the necessary documents for filing a Provisional Patent Application for the above-identified technology on behalf of The University of British Columbia. Also enclosed is Credit Card payment form PTO-2038 to cover the cost of the $80.00 application fee.

Thank you,

Sincerely,

Elaine A. Weiss, B.Sc, MBA
Technology Transfer Manager

Encl.

J·I·L·O

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Title: Thrombomodulin (THBD) haplotypes predict outcome of patients.

Inventor: Keith Walley, Vancouver, CANADA
James Russell, Vancouver, CANADA

Abstract:

The invention involves characterization of polymorphisms in the Thrombomodulin (THBD) gene that are associated with adverse outcomes in patients. Methodologies for screening haplotypes are described. THBD haplotype screening will be useful in identifying patients who would benefit from increased monitoring by healthcare professionals, and/or possible therapeutic intervention, when said patient become subject to inflammation due to systemic inflammation response syndrome (SIRS), bacterial infection, bacteraemia, sepsis, septic shock, organ dysfunction, and trauma.

Background of the Invention:

Thrombomodulin (THBD) is a critical component of the activated protein C anti-coagulant pathway. THBD is a glycoprotein receptor found on endothelial cell surfaces that forms a high affinity complex with thrombin and inhibits its pro-coagulant activities (9-11). In addition, the thrombin-TM complex activates protein C (10), which binds to protein S on cell surfaces and degrades the clotting factors V and VIII (19, 26, 30, 31). THBD also has anti-inflammatory activity, inhibiting both cytokine formation and leukocyte-endothelial cell adhesion (7).

The activation of protein C by the THBD-thrombin complex is reduced in sepsis, resulting in perturbations in the coagulatory system and disseminated intravascular coagulation (22). THBD biosynthesis has been shown to be decreased by both endotoxin and hypoxia (28, 32). Microthrombi generated in this hyper-coagulable state lead to multiple system organ failure.

There are no known associations of polymorphisms of the THBD gene with outcome in sepsis. Animal studies suggest, however, that mutations in the THBD gene may be important in the pathophysiology of sepsis. A point mutation in THBD eliminated generation of activated protein C (APC) and inhibition of thrombin, generating a prothrombothic state in homozygous mutant mice (42). The THBD gene is well characterized and a number of polymorphisms have been examined for association with thrombosis or arteriosclerosis. A C-to-A polymorphism at position -133 and a G-to-A polymorphism at position -33 in the promoter region of the THBD gene cause decreased transcription of the TM gene, and consequently decreased expression of THBD (15, 29, 33). Both polymorphisms have been associated with myocardial infarction (MI), in

Caucasian and Asian populations respectively (15, 29, 33). A G-to-A polymorphism at position 125 results in the replacement of an alanine at amino acid 25 with a threonine, and is associated with a 2-fold increased risk of MI (8). Kunz et al. detected a novel missense mutation, Arg385Ser, in an elderly woman who had suffered 3 episodes of deep venous thrombosis (DVT) (21). The mutation was found to reduce THBD expression 2-fold and the co-factor activity of THBD 4-fold (21). It is possible that polymorphisms resulting in decreased expression of thrombomodulin on endothelial cells may contribute to a hyper-coagulable state in sepsis, and may be predictive of poor outcome.

Previous studies have tested single nucleotide polymorphisms (SNPs) in putative regulatory regions or rare SNPs found to cause missense mutations for association with thrombotic and atherosclerotic disease. Haplotypes are sets of SNPs in linkage disequilibrium with one another within a gene or segment of DNA that are inherited as a single unit (1, 43). Haplotypes serve as markers for all known and unknown SNPs within a haplotype, thus a haplotype-based approach to association studies can narrow down the search for a SNP that causes a change in phenotype (1). Haplotypes can be further grouped into sets of evolutionarily related groups, or clades (41). Haplotypes within a clade differ by only a few SNPs, and the variation within a clade is much smaller than the variation between clades. Grouping haplotypes into clades increases the statistical power to associate genetic variation with a change in phenotype (41). "Tag" SNPs (tSNPs) can be selected to uniquely define a clade and serve as markers for all SNPs within haplotypes of the clade.

Systemic inflammatory response syndrome (SIRS) is characterized by increased inflammation (relative to anti-inflammatory processes), increased coagulation (relative to anti-coagulant processes), and decreased fibrinolysis (5, 6, 17, 18, 24, 39). THBD is an endothelial cell surface receptor that binds circulating thrombin and inhibits its coagulant activities. The thrombomodulin:thrombin complex activates protein C and also has downstream anti-inflammatory effects. Polymorphisms in the THBD gene may disrupt anti-coagulatory and anti-inflammatory pathways, which may be associated to adverse outcomes in SIRS.

Previous literature reports a number of SNPs in the promoter region (G-201A, G-33A) and the coding region (F127A, C1418T, and G1456T) of the thrombomodulin gene have been tested for association to the occurrence and risk of thrombotic events and cardiovascular disease (8, 15, 21, 29, 33). The 33A allele has been found to decrease promoter activity of the thrombomodulin promoter region and may be associated with altered soluble thrombomodulin serum levels and coronary artery disease, carotid atherosclerosis, and myocardial infarction. The G-201A and G1456T polymorphisms were found to be rare in patients with severe thrombophilia and possibly functionally irrelevant. The

G127A polymorphism was weakly associated with increased risk of myocardial infarction in young men when additional risk factors such as smoking were present. The C1418T polymorphism may promote formation of varicose veins, and was associated with premature myocardial infarction and coronary heart disease. It was not associated with risk of venous thromboembolism. The associations of these polymorphisms with various thrombotic events and cardiovascular disease are uncertain and there have been a number of negative studies. There have been no previous studies examining the association of thrombomodulin polymorphisms with clinical outcome in critical illness, although the protein C pathway has been found to be central to the pathophysiology of sepsis.

Using a novel haplotype-based analysis, the inventors have identified single nucleotide polymorphisms (SNPs) in the THBD gene that identify a family of THBD haplotypes (clade) that are associated with statistically significant differences in important measures of clinical outcome such as survival and organ dysfunction. The present invention describes a better strategy of predicting patients who are at a greater risk of an adverse outcome, thus enabling earlier intervention and facilitating patient-tailored therapy based on genotype.

Summary of the Invention:

The present invention is concerned with single nucleotide polymorphisms (SNPs), which form haplotypes within the thrombomodulin (THBD) gene, which are predictive of patient outcome should that patient experience inflammation. Examples of inflammation experienced by patients include, but are not limited to, systemic inflammation response syndrome (SIRS), bacterial infection, bacteraemia, sepsis, septic shock, organ dysfunction, and trauma. This invention is novel, as the respective grouping of haplotypes described in the invention predict risk of inflammation and sepsis and patient outcome much more accurately than previously identified THBD polymorphisms.

In one aspect, the present invention provides the methodology required to screen patients in order to determine those at risk of an adverse outcome following inflammation. Genetic material is collected from the patient, most commonly by isolating leukocytes from the blood, but alternatively through a variety of biopsy methods, in order that the haplotype of the THBD gene can be ascertained. Determination of the haplotype from the genetic material can be done through a variety of methods commonly described in the art, including, but not limited to sequencing, restriction fragment length polymorphism (RFLP) analysis, hybridization, oligonucleotide ligation assay, ligation rolling circle amplification, allele specific PCR, and single base-pair extension assays.

Sequence data from any of the above mentioned assays could be stored in a database for future retrieval and haplotype analysis.

In another aspect of the invention, those patients at highest risk of inflammation are the infirm, elderly, and those individuals requiring hospitalization for a variety of reasons. These at risk individuals could be screened for the THBD haplotypes associated with elevated THBD such that those individuals can benefit from increased monitoring, and possible prophylactic treatments, in order to avoid the adverse effects of inflammation.

In another aspect of the invention, patients suffering from inflammation could be screened for the THBD haplotypes associated with decreased THBD such that those individuals can benefit from increased monitoring, and possible prophylactic treatments begun in order to avoid the adverse effects of inflammation.

In another aspect of the invention, the invention provides the methodology required to determine patient outcome following collection of genetic material and haplotype determination by analysing the THBD gene, whereby the specific THBD SNPs that form the respective haplotypes are located in the sequence described in SEQ ID NO:1.

In another aspect, the invention further provides the methodology required to determine patient outcome following collection of genetic material and haplotype determination by analysing the THBD gene, whereby 5 major haplotype clades could be defined by identifying the SNP at positions 5110, 5318 and 6235 of SEQ ID NO:1.

In another aspect, the invention further provides the methodology required to determine patient outcome following collection of genetic material by analyzing the THBD gene for 5110G/5318A/6235A, 5110A/5318A/6235A 5110A/5318A/6235G or 5110G/5318A/6235G haplotypes, whereby those individuals display an adverse outcome. This outcome is due to decreased survival arising from inflammation due to organ dysfunction, SIRS, sepsis, septic shock, bacterial infection, bacteraemia or trauma.

In another aspect, the invention further provides the methodology required to determine patient outcome following collection of genetic material by analysing the THBD gene at position for the 5110A/5318C/6235A haplotype, whereby those individuals do not display as severe an adverse outcome.

The sequence positions referred to in this invention and detailed in SEQ ID NO:1 refer to the sense strand of the THBD gene. It will be obvious to a person

skilled in the art that analysis could be conducted on the anti-sense strand to determine patient outcome.

The invention further provides for kits useful in carrying out the methods of the invention.

Brief Description of the Drawings:

Figure 1. Haplotype structure of the THBD gene in Caucasians. THBD haplotypes, inferred using PHASE from available data, are illustrated in the style of Patil et al. Each column represents a polymorphic site within the THBD gene and is labelled on the left with the position in the gene. Each row represents one of the inferred haplotypes ordered by phylogenetic relationship (Figure 2). MEGA II was used to sort haplotypes into clades separated by heavier lines. Haplotypes within each clade are very similar while clades differ substantially from each other. G5110A, A5318C, and A6235G were chosen as htSNPs.

Figure 2. Evolutionary relationships of THBD haplotypes. Haplotypes were sorted into 5 clades according to evolutionary tree structure. Clades are labelled by the alleles at 5110, 5318, and 6235. Tree branch distance is % difference between haplotype sequences. (Scale bar = 2% difference).

Figure 3. 28 day mortality rates by THBD clade. The G/A/A, A/A/A, G/A/G, and A/A/G haplotype clades appeared to be associated with higher 28 day mortality rates than the A/C/A clade in 223 patients with SIRS.

Figure 4. 28 day mortality rates by THBD clade in patients with sepsis or septic shock on day one. The G/A/A, A/A/A, G/A/G, and A/A/G haplotype clades showed a stronger association with increased 28 day mortality rates in 130 patients who had sepsis or septic shock on day one of the study.

Figure 5. 28 day mortality rates associated with G/A/A, A/A/A, G/A/G, and A/A/G clades vs A/C/A clade in 130 patients with sepsis or septic shock on day one.
(A) The G/A/A, A/A/A, G/A/G, and A/A/G clades were associated with significantly increased 28 day mortality rates than the A/C/A clade in patients who had sepsis or septic shock on day one of the study ($p=0.03$).
(B) Kaplan-Meier analysis of censored mortality data showed that the G/A/A, A/A/A, G/A/G, and A/A/G clades were associated with greater mortality rates for the entire 28 day observation period ($p<0.03$).

Figure 6. DAF of cardiovascular dysfunction by THBD clade. The G/A/A, A/A/A, G/A/G, and A/A/G clades were associated with fewer DAF of cardiovascular failure (p=0.02) and fewer DAF of vasopressors (p=0.03) in patients with sepsis or septic shock on day 1 of the study.

Figure 7. DAF of respiratory dysfunction by THBD clade. The G/A/A, A/A/A, G/A/G, and A/A/G clades were associated with fewer DAF of respiratory failure (p=0.02) and fewer DAF of ventilation (p=0.008) in patients with sepsis or septic shock on day 1 of the study.

Detailed Description of the Invention:

Definitions

Allele — One of the variant forms of a gene at a particular locus, or location, on a chromosome. Different alleles produce variation in inherited characteristics such as hair color or blood type. In an individual, one form of the allele (the dominant or major one) may be expressed more than another form (the recessive or minor one).

Clade — A group of haplotypes that are closely related phylogenetically. For example, if haplotypes are displayed on a phylogentic (evolutionary) tree a clade includes all haplotypes contained within the same branch.

Genetic Material — Genetic material refers to nucleic acids, whether deoxyribonucleic acid or ribonucleic acid, isolated from cells acquired from tissue or organisms.

Genotype — Genotype refers to the genetic makeup of an organism.

Haplotype — The set of genes, comprised of one allele of each gene, which make up the genotype.

Phenotype — Phenotype refers to the observable characteristics of an organism produced by the organism's genotype interacting with the environment.

Single Nucleotide Polymorphism (SNP) — A SNP is a place in the genetic code where DNA differs from one person to the next by a single nucleotide base pair. These slight genetic variations between human beings may predispose some people to disease and explain why some respond better to certain drugs.

Methods

Patient Cohort — All patients admitted to the Intensive Care Unit (ICU) of St. Paul's Hospital were screened for inclusion. This ICU is a mixed medical – surgical ICU in a tertiary care, university-affiliated teaching hospital of the University of British Columbia. SIRS was considered present and the patients included in the study when patients met at least two of four SIRS criteria. The SIRS criteria were 1) fever (>38 °C) or hypothermia (<35.5 °C), 2) tachycardia (>100 beats/min in the absence of beta blockers, 3) tachypnea (>20 breaths/min) or need for mechanical ventilation, and 4) leukocytosis (total leukocyte count > 11,000/μL) (2). Patients were included in this cohort on the calendar day on which the SIRS criteria were met. To decrease the confounding influence of population admixture secondary to ethnic diversity on associations between genotype and phenotype, only Caucasian patients were studied.

700 consecutive critically ill patients admitted to St. Paul's Hospital ICU were screened for inclusion into our study. Of these, 600 patients (94%) met the inclusion criteria of having at least two out of four SIRS criteria. From this group, 223 patients were Caucasian and were successfully genotyped and used as our final cohort for analysis.

Clinical Phenotype — Our primary outcome variable was 28 day mortality. Secondary outcome variables were measures of organ dysfunction and of the intensity of SIRS and sepsis.

Baseline demographics that were recorded included age, gender, medical or surgical diagnosis for admission (according to APACHE III diagnostic codes(18)), and admission APACHE II score. After meeting the inclusion criteria, data were recorded for each 24 hour period (8 am to 8 am) for 28 days to evaluate organ dysfunction, SIRS, sepsis, and septic shock.

Measures of organ dysfunction – Organ dysfunction for each organ system was defined as being present during a 24-hour period if there was evidence of at least moderate organ dysfunction using the Brussels criteria (Table 1) (36). Because data were not always available during each 24 hour period for each organ dysfunction variable, we used the "carry forward" assumption as defined previously (3). Briefly, for any 24 hour period in which there was no measurement of a variable, we carried forward the "present" or "absent" criteria from the previous 24 hour period. If any variable was never measured, it was assumed to be normal.

To further evaluate cardiovascular, respiratory, and renal function we also recorded, during each 24 hour period, vasopressor support, mechanical ventilation, and renal support, respectively. Vasopressor use was defined as dopamine > 5 μg/kg/min or any dose of norepinephrine, epinephrine, vasopressin, or phenylephrine. Mechanical ventilation was defined as need for

intubation and positive airway pressure (i.e. T- piece and mask ventilation were not considered ventilation). Renal support was defined as hemodialysis, peritoneal dialysis, or any continuous renal support mode (e.g. continuous veno-venous hemodialysis). In addition, the severity of respiratory dysfunction was assessed by measuring the occurrence of acute lung injury at the time of meeting the inclusion criteria. Acute lung injury was defined as having a PaO_2/FiO_2 ratio <300, diffuse infiltrates pattern on chest radiograph, and a CVP <18 mm Hg.

Measures of the intensity of SIRS and sepsis – Each of the four SIRS criteria were recorded as present or absent during each 24-hour period. Sepsis was defined as the presence of two or more SIRS criteria plus the presence of a known or suspected infection during the 24-hour period. Cultures that were judged to be positive due to contamination or colonization were excluded. Septic shock was defined as the presence of sepsis plus significant hypotension (systolic blood pressure <90 mm Hg or the need for vasopressors) during the same 24-hour period.

Days alive and free - To assess duration of organ dysfunction and to correct organ dysfunction scoring for deaths in the 28 day observation period, we calculated days alive and free of organ dysfunction (DAF) as previously reported (4). Briefly, during each 24-hour period for each variable, DAF was scored as 1 if the patient was alive and free of organ dysfunction (normal or mild organ dysfunction). DAF was scored as 0 if the patient had organ dysfunction (moderate, severe, or extreme) or was not alive during that 24-hour period. Each of the 28 days after meeting the inclusion criteria was scored in each patient in this fashion. Thus, the lowest score possible for each variable was zero and the highest score possible was 28. A low score is indicative of more organ dysfunction as there would be fewer days alive and free of organ dysfunction.

Microbiology – Microbiological cultures were taken for any patients who were suspected of having an infection. As this is a cohort of critically ill patients with SIRS, most patients had cultures taken. Positive cultures that were suspected of having been contaminated or colonized were excluded. Positive cultures that were deemed to clinically be clinically irrelevant were also excluded. Cultures were categorized as gram positive, gram negative, fungal or other. The sources of the cultures were respiratory, gastrointestinal, skin, soft tissues or wounds, genitourinary, or endovascular.

Haplotypes and Selection of htSNPs — Using unphased Caucasian genotypic data from the Coriell registry (frompga.mbt.washington.edu), we inferred haplotypes of THBD gene using PHASE software (40). We then used MEGA 2 to infer a phylogenetic tree to identify major haplotype clades (20). Haplotypes were

sorted into clades according to this phylogenetic tree and this haplotype structure was inspected to choose "haplotype tag" SNPs (htSNPs) (12, 16). We chose 3 ht SNPs that identified 5 major haplotype clades of THBD in Caucasians. The first SNP was a G-to-A transition at nucleotide 5110 relative to the start transcription site (rs1042580), the second SNP was an A-to-C transversion at nucleotide 5318 (rs3176123), and the third htSNP was an A-to-G transition at nucleotide 6235 relative to the start transcription site (rs1962) (NCBI Thrombomodulin accession number AF495471)(SEQ ID NO. 1). These SNPs were then genotyped in our patient cohort to define haplotypes and haplotye clades.

Blood collection and processing – The buffy coat was extracted from whole blood and samples transferred into 1.5 ml cryotubes and stored at –80°F. DNA was extracted from the buffy coat using the Qiagen DNA Blood Mini Kit. The genotypic analysis was performed in a blinded fashion, without clinical information.

Genotyping — Patients' genotypes at G5110A, A5318C and A6235G were determined by real time polymerase chain reaction (PCR) assay using specific fluorescence-labeled hybridization probes in the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Inc.) as described by Livak (23). Briefly, the ABI Prism 7900HT uses a 5' Nuclease Assay in which an allele-specific probe labeled with a fluorogenic reporter dye and a fluorogenic quencher is included in the PCR reaction. The probe is cleaved by the 5' nuclease activity of Taq DNA polymerase if the probe target is being amplified, freeing the reporter dye and causing an increase in specific fluorescence intensity. Mismatched probes are not cleaved efficiently and thus do not contribute appreciably to the final fluorescent signal. An increase in a specific dye fluorescence indicates homozygosity for the dye-specific allele. An increase in both signals indicated heterozygosity. DNA from lymphocyte cell lines obtained from the Coriell Cell Repository was used to ensure the accuracy of the genotyping. The genotype of these cell lines at G5110A, A5218C and A6235 was determined using the ABI Prism 7900HT Sequence Detection system and compared to the genotype of the same cell lines determined by direct sequencing, given at *www.pga.mbt.washington.edu* (37).

Statistical Analysis – A cohort study design was used. A chi-squared test was used to test for an association between 28-day mortality and haplotype clades. This initial analysis identified the A/C/A haplotype clade as being distinct from all other clades. For subsequent analysis differences in clinical outcomes were compared between the A/C/A haplotype clade versus all other haplotypes combined. Rates of dichotomous outcomes (28-day mortality, sepsis and shock at onset of SIRS) were compared between the 2 groups of haploty[e clades · using a chi-squared test. Differences in continuous outcome variables between

the A/C/A haplotype clade and all other haplotype clades were tested using ANOVA. Baseline descriptive characteristics were compared using chi-squared test and ANOVA where appropriate. 28-day mortality was further compared between the A/C/A haplotype clade and all other haplotype clades while adjusting for other confounders (age, sec, and medical vs. surgical diagnosis) using a Cox regression analysis in addition to a Kaplan-Meier analysis. Haplotype clade relative risk was calculated. Genotype distributions were tested for Hardy-Weinberg equilibrium (14). We report the mean and 95% confidence intervals. Statistical significance was set at $p<0.05$. The data was analyzed using SPSS 11.5 for Windows and SigmaStat 3.0 software (SPSS Inc., Chicago, IL, 2003).

Discussion

We hypothesized that haplotype clades of thrombomodulin are associated with outcome from sepsis. To test this hypothesis we determined the haplotype structure of the thrombomodulin gene using publicly available data (from pga.mbt.washington.edu) (37). We then used cladistic analysis to group these haplotypes into related clades (Figures 1 and 2) (20) and subsequently determined a minimum set of "tag" SNPs (tSNPs) (Figure 1) that defined all 5 major haplotype clades of the thrombomodulin gene (12, 16, 41). We then tested for the association of these haplotype clades with 28 day mortality and organ dysfunction in a cohort of critically ill adults who had SIRS (35).

We found that the thrombomodulin haplotype clade defined by 5110A/5318C/6235A was significantly associated with decreased 28-day mortality ($p=0.03$), less organ dysfunction (cardiovascular, $p=0.02$; respiratory, $p=0.02$; hematologic system, $p=0.04$; neurologic, $p=0.02$, hepatic, $p=0.04$), and less sepsis ($p<0.02$) in patients who had sepsis or septic shock upon admission to the study. We conclude that the clades marked by 5110A/5318A/6235A, 5110A/5318A/6235A, 5110A/5318A/6235G, and 5110G/5318A/6235G are markers of adverse outcome in critically ill patients with sepsis or septic shock, and can be used to predict statistically significant differences in important measures of clinical outcome.

To our knowledge there are no other reported associations of polymorphisms of thrombomodulin with outcome in systemic inflammatory response syndrome and sepsis. A G-to-A polymorphism at position -33 in the promoter region of the thrombomodulin gene is particularly frequent in Asians, and is associated with coronary artery disease (CAD), myocardial infarction. The thrombomodulin G-33A polymorphism is near a consensus sequence for transcription control elements, and reporter gene assays have shown that the -33A allele decreases promoter activity. Interestingly, it has been found that in CAD patients homozygous -33G allele soluble thrombomodulin levels increased with the extent

of CAD. In CAD patients who were homozygous or heterozygous for the -33A allele, levels of soluble thrombomodulin did not change with the extent of vessel disease.

Poor outcome from infection has been shown to be highly heritable. Genotype has been shown to contribute substantially to outcome in sepsis (25, 27, 34). The genetic contribution to death from sepsis exceeds the inherited genetic contribution to cancer risk of death by many fold and even exceeds the genetic contribution to cardiovascular disease risk (39). Genetic polymorphisms in molecules of the protein C pathway may cause inter-individual variation in the response to infection, and may be predictive of patients' outcome from sepsis. If our current findings hold up in larger populations then we could conclude that the haplotype clade marked by the 5110A, 5318C, and 6235A alleles of the thrombomodulin gene is a marker of improved outcome in critically ill patients. Conceivably this knowledge would be helpful in identifying critically ill patients who may benefit from directed therapy, specifically patients who did not carry a haplotype from within the clade marked by 5110A, 5318C, and 6235A. Clinicians would use this knowledge to identify critically ill patients at risk for adverse outcome to design patient-tailored therapy based on genotype.

As recombinant human Activated Protein C (rhAPC) and other anti-coagulatory treatments become standard care in sepsis, it is essential to test genetic variants of coagulation system genes for association to outcome in sepsis in order to incorporate genotype into the design of patient-tailored therapy. Currently, rhAPC is indicated only for patients with severe sepsis (APACHE II $\geq$ 25). If patients at risk by genotype for poor outcome from sepsis could be identified prospectively, rhAPC could be administered to these patients earlier and potentially lower mortality from sepsis even further.

Drug companies could use knowledge of genetic risk factors for poor outcome from sepsis to target their randomized control trials to patients who would benefit most from drug therapies with few side effects based on their genotype. Our association of haplotypes of thrombomodulin with poor outcome in critically ill patients would be particularly useful in trials of drugs that modify coagulation and fibrinolysis such as activated protein C, protein C concentrate, tissue factor pathway inhibitor (TFPI), heparin, tissue plasminogen activator and other drugs in development may be more beneficial in patients identified to be at increased risk of death and organ dysfunction.

Examples:

252 consecutive critically ill patients admitted to the ICU of St. Paul's Hospital were screened for inclusion. Of these, 223 Caucasian patients were successfully genotyped and make up the cohort of this study.

Example 1
Haplotype clade deduction

We were able to infer haplotypes from complete sequencing of THBD for 23 Caucasians in the Coriell Cell Repository (37) using PHASE software (40), and identified two major haplotype clades using MEGA2 software (20) (Figures 1 and 2). These 5 clades could be resolved by genotyping three htSNPs: G5110A, A5318C and A6235G, in our 223 patient cohort. The 5110G/5318A/6235A (G/A/A) haplotype clade occurred with a frequency of 36.3%, the A/A/A haplotype clade occurred with a frequency of 22.4%, and a/A/G haplotype clade occurred with a frequency of 21.5%, the A/C/A haplotype clade occurred with a frequency of 18.4%, and the G/A/G haplotype clade occurred with a frequency of 1.3%. The genotypes of all three htSNPs were similar to frequencies deduced from other available Caucasian data(37) and were in Hardy-Weinberg equilibrium (Table 2) (14).

For the 223 successfully genotyped individuals of the cohort of Caucasian patients who had at least 2 of 4 SIRS criteria, no haplotype clade of THBD was significantly associated with a difference in age, gender or severity of illness at the time of admission to the study (as estimated by the APACHE II score) (Table 2). By chance, the A/C/A haplotype clade was associated with a higher proportion of surgical diagnoses for admission to the ICU (Table 2).

Example 2
Haplotype patient outcome

Upon preliminary analysis by ANOVA, the A/C/A haplytype clade appeared to be associated with a lower rate of 28-day mortality than the G/A/A, A/A/A, G/A/G, and A/A/G haplotype clades (Figure 3). This trend was stronger in patients who had sepsis or septic shock at the time they were admitted to the study (Figure 4). We subsequently chose to compare the 4 haplotype clades which were associated with increased rates of 28-day mortality as a group to the A/C/A haplotype clade. Further analysis was limited to the 130 patients who had sepsis or septic shock at the time they were admitted to the study. The average APACHE II score of these patients was 21.4 ± 7.9. There was no difference between clades in the proportion of medical vs. surgical diagnoses in this subgroup of patients.

In patients who had sepsis or septic shock at the time they were admitted to the study, the G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was associated with significantly greater 28-day mortality than the A/C/A haplotype clade ($p=0.03$) (Figure 5a). Kaplan-Meier analysis of 28-day mortality verified that the G/A/A, A/A/A, G/A/G and A/A/G haplotype clades were significantly associated

with increased rates of mortality over the entire 28-day observation period (p<0-.03) (Figure 5b). A Cox multiple regression model demonstrated that the G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was an independent predictor of mortality after adjusting for other predictors of survival (age, sex, medical vs surgical diagnosis at admission) (p<0.03) (Table 4).

The G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was associated with a more vigorous inflammatory response. In our entire 223 patient cohort, the G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was associated with fewer DAF of 4 of 4 (20.6 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs. 23.1 days for the A/C/A clade, p=0.05), 3 of 4 (20.3 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs. 22.7 days for the A/C/A clade, p=0.06) and 2 of 4 SIRS criteria (19.9 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs. 22.4 days for the A/C/A clade, p=0.05). In the subgroup of 130 patients who had sepsis or septic shock upon admission to the study the G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was even more strongly associated with fewer DAF of 4 of 4 (20.0 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs. 23.9 days for the A/C/A clade, p=0.01), 3 of 4 (19.7 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs. 23.1 days for the A/C/A clade, p=0.02) and 2 of 4 SIRS criteria (19.1 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs 23.0 days for the A/C/A clade, p=0.01).

The G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was associated with fewer days alive and free of multiple-system organ failure. The G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was significantly associated with fewer DAF of cardiovascular failure (p=0.02), and the need for more cardiovascular support as measured by fewer DAF of vasopressors (p=0.03) (Figure 6). The G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was associated with fewer DAF of respiratory failure (p=0.02) and fewer DAF of ventilation (p=0.008) (Figure 7). The G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was also associated with fewer DAF of hematologic system failure (23.8 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs. 26.5 days for the A/C/A clade, p=0.04) fewer DAF of neurologic dysfunction (18.4 for the G/A/A, A/A/A, G/A/G and A/A/G clade vs. 22.1 days for the A/C/A clade, p=0.02), and fewer DAF of hepatic dysfunction (18.1 days for the G/A/A, A/A/A, G/A/G and A/A/G clade vs. 21.6 days for the A/C/A clade, p=0.04).

When analyzed individually, there was no significant association between the htSNPs G5110A, A5318C, or A6235G and 28-day mortality or multiple system organ failure.

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TABLE 1
Brussels Organ Dysfunction Scoring System

5

ORGANS	Free of Organ Dysfunction		Clinically Significant Organ Dysfunction		
	Normal	Mild	Moderate	Severe	Extreme
Cardiovascular Systolic BP (mmHg)	>90	≤90 Responsive to fluid	≤90 Unresponsive to fluid	≤90 plus pH ≤7.3	≤90 plus pH ≤7.2
Pulmonary P_aO_2/F_IO_2 (mmHg)	>400	400-301	300-201 Acute lung injury	200-101 ARDS	≤100 Severe ARDS
Renal Creatinine (mg/dL)	<1.5	1.5-1.9	2.0-3.4	3.5-4.9	≥5.0
Hepatic Bilirubin (mg/dL)	<1.2	1.2-1.9	2.0-5.9	6.0-11.9	≥12
Hematologic Platelets ($x10^5/mm^3$)	>120	120-81	80-51	50-21	≤20
Neurologic (Glascow Score)	15	14-13	12-10	9-6	≤5
Round Table Conference on Clinical Trials for the Treatment of Sepsis Brussels, March 12-14, 1994 (38).					

TABLE 2. Genotype Frequencies and Allele Frequencies for three htSNPs of thrombomodulin in a Cohort of 223 Critically Ill Adults who had SIRS

	Genotype Frequencies			Allele Frequencies		p *
	GG	GA	AA	G	A	
G5110A	17%	42%	41%	38%	62%	0.119
	AA	AC	CC	A	C	
A5318C	67%	29%	4%	82%	18%	0.514
	AA	AG	GG	A	G	
A6235G	62%	31%	7%	77%	23%	0.086

* exact test of Guo and Thompson to test for Hardy-Weinberg equilibrium (13)

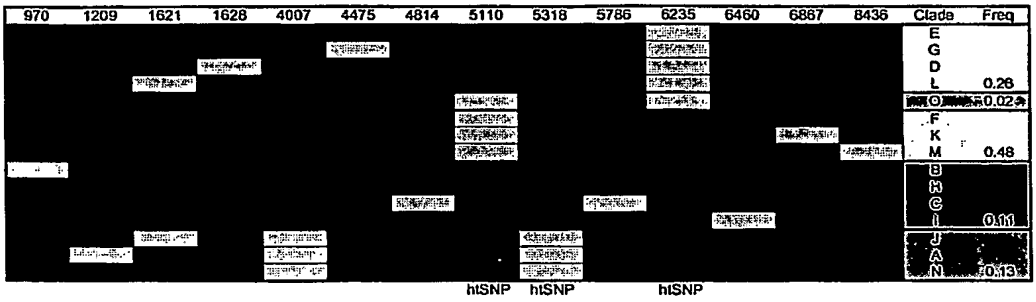
TABLE 3. Baseline Characteristics of 223 critically ill patients with SIRS by thrombomodulin haplotype clade

Haplotype Clade	Frequency	Mean Age	Gender (% Male)	Diagnosis for admission (% Surgical)	Mean APACHE II
G/A/A	36%	59	60%	26%	18
A/C/A	18%	59	61%	44%	19
A/A/A	22%	59	69%	23%	20
G/A/G	1%	69	50%	17%	19
A/A/G	22%	61	68%	33%	20
p		NS	NS	0.02	NS

TABLE 4. Cox Proportional Hazard Analysis – Hazard Ratios for Mortality

Covariate	Hazard Ratio	95% CI	p
Female sex	0.63	0.41-0.98	0.04
Age	1.00	0.99-1.02	0.45
Surgical Diagnosis	0.77	0.50-1.17	0.21
G/A/A, A/A/A, G/A/G, or A/A/G	1.95	1.05-3.57	0.03

Figure 1. Haplotype structure of the thrombomodulin gene in Caucasians



5

Figure 2. Unrooted phylogenetic tree of thrombomodulin haplotypes in Caucasians.

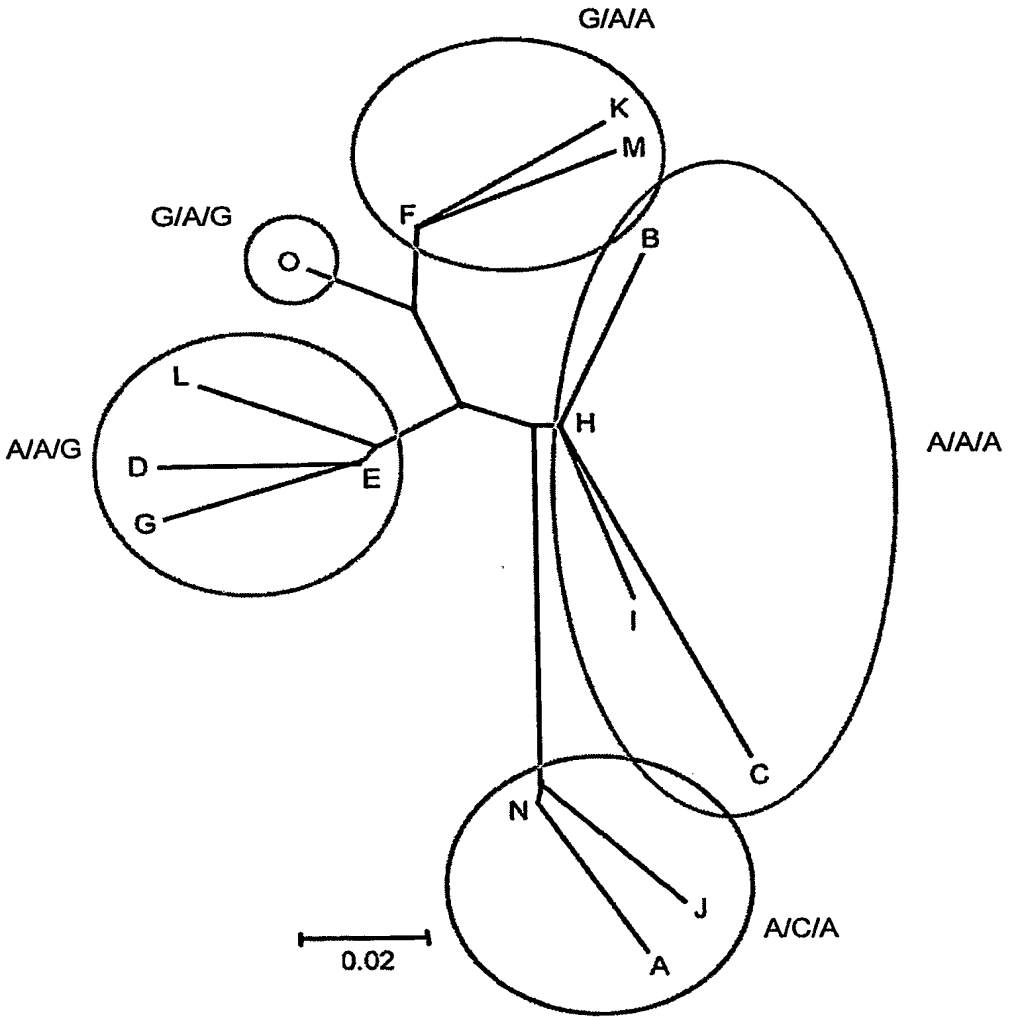


Figure 3. 28-day Mortality in 223 critically ill patients with SIRS by haplotype clade

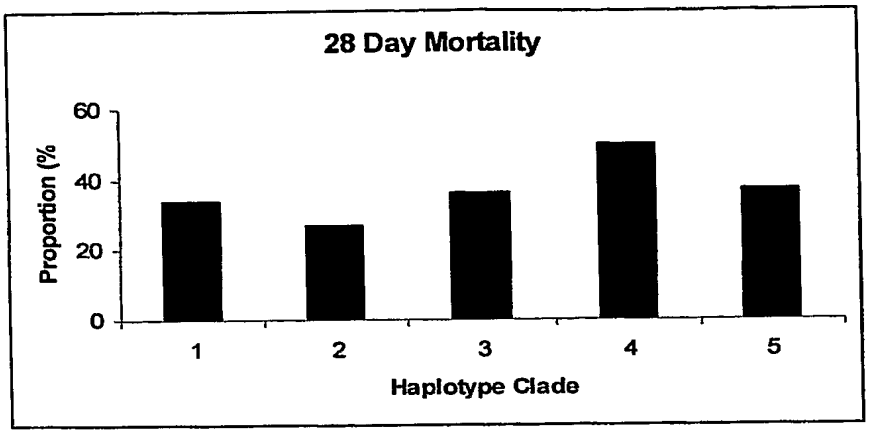


Figure 4. 28 day Mortality in 130 critically ill patients with sepsis or septic shock on day one of observation.

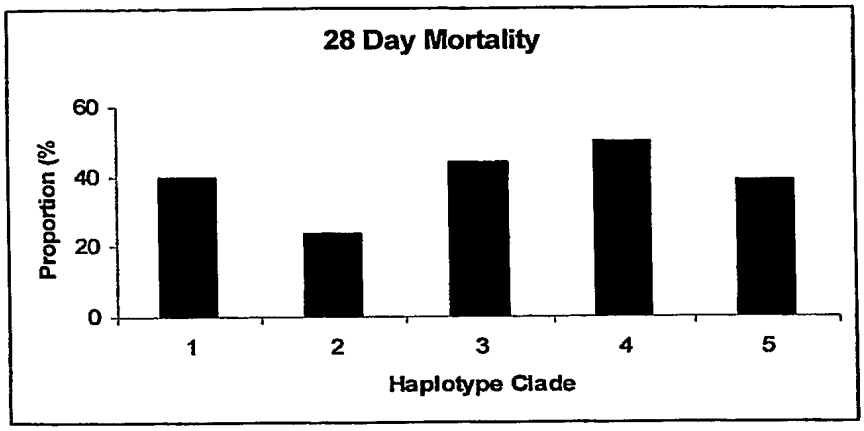


Figure 5a. 28 day Mortality in 130 patients with sepsis or septic shock on day one of the study.

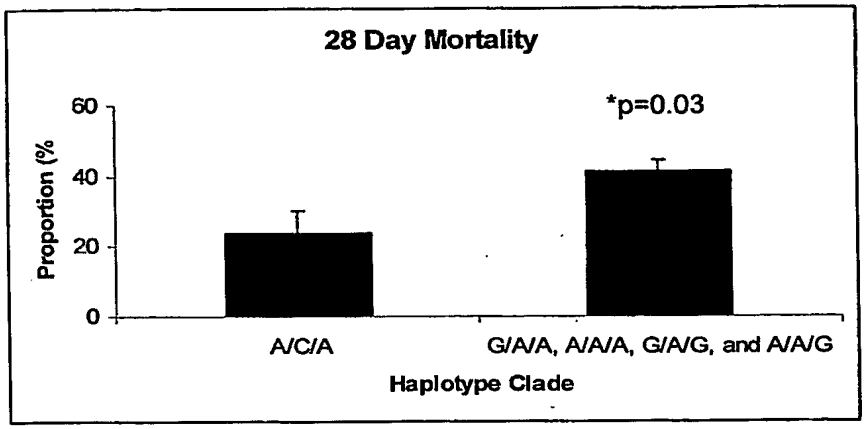


Figure 5b. Kaplan-Meier survival analysis in 130 patients with sepsis or septic shock on day one of the study.

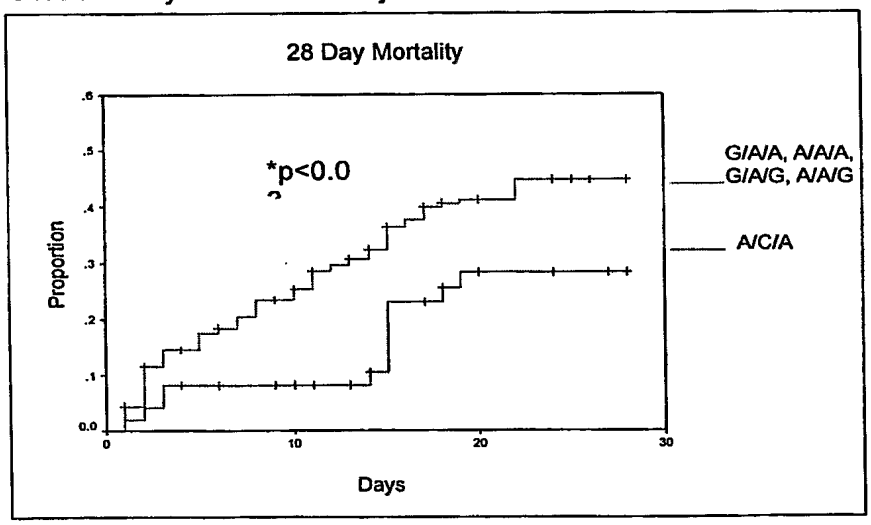


Figure 6. Days alive and free of Cardiovascular dysfunction in 130 patients with sepsis or septic shock on day one of the study.

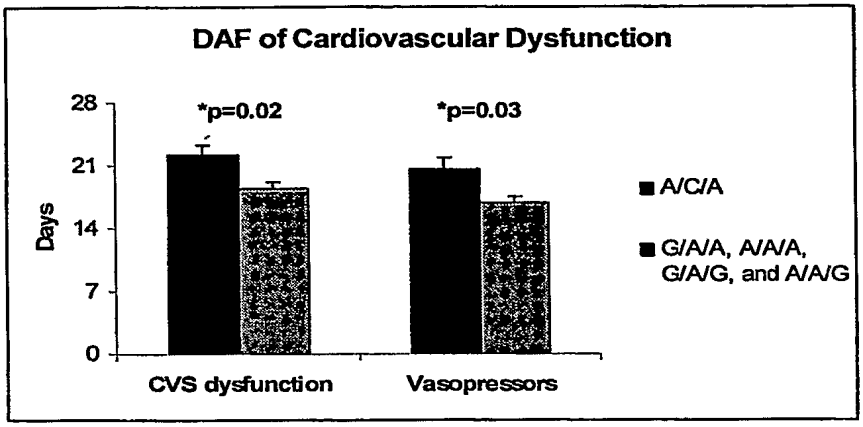
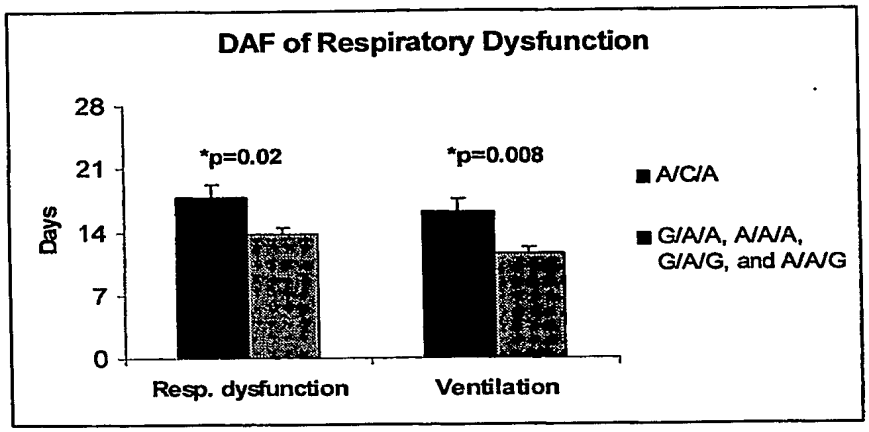


Figure 7. Days alive and free of Respiratory dysfunction in 130 critically ill patients with sepsis or septic shock on day one of study.



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SEQ ID NO.1

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A5318C
TTACTTATTTTTGACAGTGTTGAAAATGTTCAGAAGGTTGCTCTAGATTG[A/C]GAGA
AGAGACAAACACCTCCCAGGAGACAGTTCAAGAAAGCTTCAAACTG

A6235G
TGAGATGCATGGAGGGCTGCCCTGTACCCCAGCACTTGTGTTGTCTGGTG[A/G]TG
GCACCATCTCTGATTTTCAAAGCTTTTTCCAGAGGCTATTATTTTCAC

SEQ ID. NO. 2

AF495471.1 Thrombomodulin (human)

```
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